The role of mitochondrial ROS in the aging brain

Rhoda Stefanatos and Alberto Sanz*

Institute for Cell and Molecular Biosciences, Newcastle University Institute for Ageing, Newcastle University, Newcastle upon Tyne NE4 5PL, United Kingdom.

*Corresponding author: Alberto Sanz. Institute for Cell and Molecular Biosciences, Newcastle University Institute for Ageing, Newcastle University, Newcastle upon Tyne NE4 5PL, UK. Tel: +44(0)191 248 1221; fax: +44(0)191 248 1101 E-mail: alberto.sanz@newcastle.ac.uk

Abstract

brain is the most complex human organ, consuming more energy than any other tissue in proportion to its size. It relies heavily on mitochondria to produce energy and is made up of mitotic and post mitotic cells that need to closely coordinate their metabolism to maintain essential bodily functions. During ageing, damaged mitochondria that produce less ATP and more Reactive Oxygen Species (ROS) accumulate. The current consensus is that ROS cause oxidative stress, damaging mitochondria, resulting in an energetic crisis that triggers neurodegenerative diseases and accelerates ageing. However, in model organisms increasing mitochondrial ROS (mtROS) in the brain extends lifespan, suggesting that ROS may participate in signalling that protects the brain. Here we summarize, the mechanisms by which mtROS are produced at the molecular level, how different brain cells and regions produce different amounts of mtROS and how mtROS levels change during ageing. Finally, we critically discuss the possible roles of ROS in ageing as signalling molecules and damaging agents addressing whether age associated increases in mtROS are a cause or a consequence of aging.

Key words: aging, brain, mitochondria, ROS, signalling

Abbreviations: coenzyme Q (CoQ), electron transport chain (ETC), hiROS (high Reactive Oxygen Species), loROS (low Reactive Oxygen Species), mitochondrial Reactive Oxygen Species (mtROS), monoamino oxidase (MAO), xanthine oxidase (XO)

1. Introduction: The importance of the brain in the aging process

The ageing process leads to the indiscriminate loss of physiological functions across an organism. As the human population continues to live longer, many age associated diseases are becoming more prevalent, placing an ever growing burden on health and social systems. Understanding how and why we age is vital if we are to find ways to live better for longer. To do this we need to examine the organs and organelles which play key roles in physiology and the aging process.

Many of our body's physiological functions are set or controlled by our brains. The brain is the most complex organ in the human body. It is the centre of our nervous system and orchestrates many processes required for homeostasis and physical actions. The brain is made up of 3 distinct regions, the cerebrum, cerebellum and brain stem and broadly two cell types, neurons and glia. These cell types can further be divided into specific types of neuron and glial cells, each with a specific role and biology [1]. The brain holds our consciousness and the key to who we are, and as such is irreplaceable. It plays a central role in physiology and metabolism and may be at the centre of aging. Understanding how the human brain ages and identifying interventions to delay or reverse this process may be crucial for the delay or reversal of whole body healthy aging.

To carry out its many autonomous and non-autonomous functions the brain is highly metabolically active, using nearly a quarter of the body's total consumption of glucose and oxygen. This high level of oxygen consumption can lead to an increase in the production of Reactive Oxygen Species (ROS). As set out by the free radical and mitochondrial free radical theories of aging, increased levels of ROS and the resulting oxidative damage correlate highly with aging [2]. One of the major sources of ROS in the brain are mitochondria, referred to as mitochondrial ROS (mtROS). Mitochondria play a role in many important cellular functions including the production of ATP via oxidative phosphorylation which also produces ROS. ROS, a collective title given to a whole host of reactive molecules, have for the most part been associated with negative effects, reviewed here [3]. However, in recent years the essential role of ROS in signalling has been uncovered[4]

Alterations in mitochondria and mitochondrial function have long been considered hallmarks and potential drivers of aging [5]. In the aging brain, mitochondrial function is reduced while the number of damaged mitochondria and ROS accumulate [6]. This also correlates with increased levels of products of oxidative damage such as 8-OH-dG and mutation load in mtDNA [6]. The increase in oxidative damage to mtDNA, mitochondrial lipids and proteins correlates with the accumulation of less functional and damaged organelles [5]. Given that the accumulation of damaged mitochondria is a universal hallmark of aging, understanding this phenomenon is essential for anti-aging interventions. Moreover, it is also likely that the dysregulation of ROS production as a function of age is linked to this.

In this review, we will explore the connection between mtROS, their role in brain physiology and aging. For the sake of space, we will focus on physiological aging of the brain i.e. that which occurs in the absence of diagnosed neurodegeneration. We will examine the nature, site and level of mtROS production in regards to cellular signalling, oxidative stress, aging and the rate of aging. Most importantly we will examine how disruption of site and species specific ROS signalling vs non-specific ROS produced from damaged mitochondria or elsewhere contributes to aging in the brain. This is particularly of interest given the failure of many antioxidant therapies to delay or reverse

aging, despite the age-related increased in oxidative damage and many examples were boosting mtROS does not have a negative impact on longevity [7, 8].

2. How and where are ROS produced in the brain?

2.1 Importance of detecting ROS *in vivo* to study ROS signalling.

For a long time ROS were considered a single entity with the level of ROS considered the only factor important in determining their physiological effects. It was during this time that the Mitochondrial Free Radical Theory of Aging was the most widely accepted explanation for aging and antioxidant therapies were seen as the "Holy Grail" for lifespan extension. Nowadays however, it is well accepted that ROS are essential components of the cellular signalling machinery, playing essential roles in cellular differentiation and maintaining homeostasis [4]. Moreover, we know that each distinct ROS has different properties. Less reactive oxygen species (loROS), such as superoxide and particularly hydrogen peroxide, participate in cellular signalling whereas highly reactive oxygen species (hiROS), such as the hydroxyl radical or peroxynitrite, can damage all types of biological molecules resulting in oxidative damage [9].

There is increasing evidence that mtROS levels are strictly controlled at the levels of generation and neutralization[10]. Controlling mtROS at the point of generation implies regulation of the type of ROS and when, where and how much is produced. Similarly, controlling levels of mtROS via neutralization requires selection of an antioxidant system as well as knowledge of when and where to act. Strikingly, cells have only developed specific enzymatic systems to scavenge loROS e.g. superoxide dismutases (against superoxide), peroxiredoxins and glutathione peroxidases (against hydrogen peroxide). This suggests that hiROS most likely originate from loROS (e.g. by Fenton and Haber-Weiss reactions) and as such preventing their generation is more effective than developing pathways to effectively neutralize hiROS.

Until recently, ROS measurements carried out in isolated mitochondria were the preferred method to study ROS. The majority of studies using this approach have reported a negative correlation between extra-mitochondrial generation of hydrogen peroxide and lifespan (reviewed in [2]). Studies in isolated mitochondria, sub-mitochondrial particles or isolated complexes have many caveats (summarized in [8]), but one big advantage is the high resolution, allowing interrogation of where and how ROS are being generated. New tools and methods have been developed to study levels of superoxide, hydrogen peroxide, peroxynitrite etc. both *ex vivo* and *in vivo* (see [11]). This has allowed the measurement of ROS in more physiological conditions with the disadvantage that they lack the resolution of *in vitro* studies. As such, it is very difficult to determine exactly where and by which mechanisms ROS are generated. These are not the optimal conditions since studying the role of ROS in cellular signalling requires examining four different parameters: when, where, how much and which type of ROS is produced.

A way to increase resolution is to use specific inhibitors of the electron transport chain (ETC) in a similar way to *in vitro* studies. Although this approach is relatively easy to implement in cells in culture, it is much more difficult to deliver inhibitors at active concentrations *in vivo*. Alternatively, it is possible to manipulate the activity of the ETC using genetic manipulation via mutation, knock-down of specific respiratory complex subunits that will prevent assembly of the respiratory complexes [12] or expression of alternative respiratory enzymes to regulate the redox state of the ETC [13]. For example, allotopic expression of an alternative oxidase (AOX) that reduces oxygen to water with

electrons from ubiquinol, allows the maintenance of the ETC in an oxidized state, preventing electron leak and ROS production in fruit flies [14]. Conversely, it is possible to increase the redox state of the ETC by allotopically expressing alternative NADH dehydrogenase internal 1 (NDI1) that reduces ubiquinone to ubiquinol. This increases electron leak and ROS production in fruit flies [14]. Finally, novel antioxidant molecules that prevent electron leak from specific sites within the respiratory complexes have been developed and tested in human cells as well as mouse and fly models [15, 16]. S1QELs (suppressors of site IQ electron leak) prevent the generation of ROS by CI [16], whereas S3QELs (selective suppressors of site IIIQo electron leak) prevent the generation of ROS by CIII[15]. These molecules do not alter the function of the complex and therefore do not alter mitochondrial respiration and thus are extremely useful to study the role of ROS in signalling.

2.2 Production of ROS at the molecular level: the mitochondrion.

There are up to 11 distinct sites where ROS can be produced in isolated mitochondria [17]. However, only three of these sites have been shown to be relevant for ROS production *in vivo*: respiratory complex I (CI *aka* NADH:ubiquinone oxidoreductase), complex II (CII *aka* succinate dehydrogenase) and complex III (CIII *aka* Ubiquinol:cytochrome c reductase) (Figure 1). Surprisingly, respiratory complex IV (CIV *aka* cytochrome c oxidase) which reduces oxygen to water does not produce any ROS, demonstrating that electron leak, and ROS production, can be prevented in the ETC. ROS produced by CII are important for the development of certain types of cancer [18], while ROS produced by CI and CIII have been shown to regulate several physiological and pathological processes ranging from cellular differentiation to damage during ischemia/reperfusion [4, 19]. Interestingly, the topology of ROS production for CI and CIII is different. All ROS produced by CI are directed to the mitochondrial matrix, whereas ROS produced by CIII are split between the intermembrane space and the matrix [20]. This distinction could be an important in determining why ROS produced at CI and III have different downstream effects.

ROS produced by CIII are required to trigger the hypoxia response and are instrumental in cellular differentiation of human adipocytes for example (reviewed in [21]). While ROS produced by CI are instrumental for the differentiation of myocytes to myotubes, oxygen sensing by cells of the carotid body, organization of the ETC, oxidative damage caused during ischemia-reperfusion, metabolic reprogramming of macrophages in response to bacterial infection and regulation of lifespan (reviewed in [8]). CI can produce ROS in both the forward and reverse direction. Interestingly, all these functions are performed when ROS are produced by reverse electron transport (RET)[8]. RET occurs when the pool of CoQ becomes over-reduced with electrons and the reduced form (ubiquinol) transfers electrons back to CI [22]. A role for RET-ROS signalling as a communication system between mitochondria and the rest of the cell is plausible as RET can provide information about how much ATP is being synthesized (since RET is influenced by changes in the proton motive force) and electron flow (as it is also sensitive to changes in the redox state of the CoQ pool). Indeed, through RET a significant amount of ROS are produced that can be used to transmit information.

The fact that ROS produced by CI and CIII activate distinct and specific cellular processes supports the existence of ROS signalling specific to each complex. Both CI and CIII would be important generators of site-specific ROS. As we will discuss below, deregulation of ROS generation by CI and CIII as well as other as yet uncharacterized generators of site-specific ROS may be an important contributor to aging.

2.3 ROS Production at the molecular level: extra-mitochondrial sites.

mtROS are considered the key population of ROS involved in the aging process as it is assumed that they are continually generated as a result of mitochondrial respiration [23]. However, enzymes located out with the mitochondrion are also able to produce ROS. A non-exhaustive list includes, NADPH oxidases (NOXs), xanthine oxidase, monoamine oxidase and peroxisomal enzymes (Figure 1).

After the mitochondrial respiratory complexes, NOXs are probably the major enzyme group producing ROS. In fact, they are the only family of enzymes whose specific function is ROS production (either superoxide or hydrogen peroxide) [24]. NOXs participate in various signalling pathways instrumental for the regulation of growth and inflammation, while their importance in aging and age-related diseases is supported by several studies [25]. There is a continuous "crosstalk" between NOXs and mtROS resulting in the modification of cellular ROS levels [26]. Other ROS generators such as xanthine oxidase (XO) [27, 28] or monoamino oxidase (MAO) [29], located in the outer membrane of the mitochondrion facing towards the cytosol, produce more ROS during aging. Increased ROS production by XO in the context of the aging brain is not clear, but increased ROS production by MAO has been linked with neuronal cell death in the substantia nigra [30].

Finally, in addition to the mitochondria other organelles have to deal with significant amounts of ROS and how they do this influences the total ROS levels in the cell. As you would expect this has consequences for redox signalling and can trigger oxidative stress. The peroxisome is one of the most important generators and detoxifiers of hydrogen peroxide in the cell [31]. Accordingly, loss of redox homeostasis within peroxisomes correlates with increased levels of hydrogen peroxide and the induction of cellular senescence [32]. Lysosomes are instrumental for turnover of damaged proteins and organelles. However, the unintended release of lysosomal content such as free metals (e.g. iron) and free radicals can promote oxidative damage [33]. Age-related accumulation of undigested lysosomal material in the form of lipofuscin is considered the best biomarker of aging [33].

In this review, we will focus mainly on mtROS. However, it is important to keep in mind that there are other sites in the cell where ROS can be produced. Those referred to above are important as they can generate site-specific ROS, participating in signalling (e.g. NOXs), or unspecific ROS, increasing oxidative damage (e.g. lysosomes). As we will discuss, deregulation of the "crosstalk" between ROS generators could underlie the age-related increase in mtROS observed during aging.

2.4 mtROS Production at the cellular level: mtROS generation in Neurons vs Glia.

Mitochondria have diverse shapes, compositions and operate differently depending on how much ATP needs to be generated and which of its supplementary functions are required [34, 35]. For example, heart and liver mitochondria have distinct lipid and protein compositions [34, 36], and accordingly respire and produce ROS at different rates [36]. This is expected since heart mitochondria need to continually supply high levels of ATP to satisfy the high energetic demand of the heart. Even within a cell, mitochondria can be diverse depending once again on the requirement of ATP and any additional functions. This is particularly true in highly polarized cells, such as neurons, where mitochondria develop specialized functions depending on the region of the cell in which they are located. For example, synaptic and non-synaptic mitochondria have different protein compositions, respiration rates and generate different amounts of ROS [37].

Neurons and glia use different metabolic pathways to produce energy. Astrocytes are highly glycolytic, while neurons require oxidative phosphorylation. Glucose is mainly consumed by glia that "feed" neurons with lactate and other small metabolites which are oxidized by neuronal mitochondria to obtain energy [38]. Given their metabolic differences, it is not surprising that neuronal and glial mitochondria produce different amounts of ROS. In a comprehensive study, Lopez-Fabuel and col. [39] demonstrated that in rats and mice CI specifically produces more ROS in glia than in neurons. Using an elegant combination of *in vitro*, *ex vivo* and *in vivo* approaches, the authors show that CI is incorporated less into respiratory supercomplexes as a result of a limitation of CIII in glia [39]. The increase of "free" CI in glia could explain why glia produces more ROS. Interestingly, modulating the levels of a single CI subunit, NDUFS1, they were able to increase or decrease mtROS in neurons and glia. For example, overexpression of NDUFS1 in glia promoted incorporation of CI into supercomplexes, reducing ROS levels [39]. Conversely, knock down of NDUFS1 in neurons increased mitochondrial ROS production. In the future, it would be interesting to determine if there are differences in CI incorporation into supercomplexes between mitochondria within a cell (e.g. synaptic versus non-synaptic mitochondria) and the effect on the rate of ROS production.

Respiratory complexes are organized into super structures known as supercomplexes [40]. Recent work has shown that the incorporation of respiratory complexes into supercomplexes is modified in response to changes in the proportion of different "fuels" or substrates that feed mitochondria (e.g. pyruvate, fatty acids, amino acids, etc) [41]. For example, CI is predominantly part of the so called "respirasome" (the super-structure made by CI in association with CIII and CIV) when pyruvate is used to obtain energy in the mitochondria, whereas it is "released" from the respirasome when fatty acids are used as a substrate [41] or glucose is oxidized via anaerobic glycolysis to obtain energy [39]. Interestingly, both the levels of CI and its assembly into supercomplexes are determined by levels of ROS and specifically by ROS produced via RET at CI [42]. Both *in vitro* and *ex vivo* experiments support the idea that to reduce ROS generation CI must be incorporated into supercomplexes [39, 43, 44]. Disruption of mitochondrial supercomplexes would increase electron leak (and ROS production) and as a result the rate of aging [45]. In support of this idea, in aged rat brains supercomplexes are disorganized [46], which could explain why their mitochondria produce more ROS [47].

2.5. ROS production in different regions of the brain.

The brain is particularly sensitive to oxidative damage due to its high rate of oxygen consumption, the abundance of polyunsaturated fatty acids prone to oxidation and the increased concentration of transition metals such as iron or copper. Due to the heterogeneous nature of the brain which includes several cell and sub cell types certain areas of the brain are more sensitive than others to changes in oxidative stress [48] or levels of ROS [49]. It is important to highlight that ROS can cause reversible redox modifications (mainly in proteins e.g. reversible oxidation of specific cysteines) as well as irreversible oxidative damage, i.e. causing structural damage in biomolecules. Therefore neurons, and other cell types, can be more susceptible to changes in ROS levels because they are more sensitive to oxidative damage or less resilient to alterations in ROS signalling. Understanding how different cells, including different types of neurons and glia, respond to changes in ROS is instrumental for designing therapies that prevent oxidative stress without interrupting ROS signalling.

Neurons and glia have diverse sizes, morphologies and metabolic features (see above). Despite producing fewer mtROS, neurons are more sensitive than glia to oxidative stress because they have fewer antioxidants [50]. In addition, we must keep in mind that neurons are post mitotic cells that are rarely replaced during the lifetime of the individual. This last feature makes neurons more sensitive to alterations in cellular homeostasis including changes in redox state. Different types of neurons also show differences in topology, myelination and the number of synaptic connections, all of which can alter their sensitivity to changes in ROS levels. This phenomenon is known as selective neuronal vulnerability [51]. In general, large neurons with long axons are more likely to die in response to increased oxidative stress [51]. Additionally, neurons which produce more ROS are those most sensitive to oxidative stress [52]. Some areas of the brain such as the hippocampus, substantia nigra, amygdala and frontal cortex are more sensitive to oxidative stress. Within these areas some neurons are more sensitive than others, i.e. CA1 neurons in the hippocampus or neurons of pars compacta in the substantia nigra [51]. In response to severe hypoglycaemia, ROS are most increased in hippocampus and striatum [53]. Coincidentally, these are the two areas that accumulate the highest level of protein carbonyls during aging [54]. The hippocampus is also the most sensitive area to damage due to ischemia-reperfusion [55] and where oxygen consumption is found to be most decreased during aging [56].

The existence of all this regional and cellular heterogeneity is an important reminder that ROS can result in neurodegeneration (or glial cell death) in certain areas of the brain but not in others. Similarly, certain areas or cells are affected by increases in oxidative stress whereas others will be damaged by alterations in redox signalling. Conversely, in certain areas the age-related increase in ROS could be a protective mechanism to stimulate activation of quality control systems via mitohormesis (see section 3), while in others an increase in unspecific ROS could cause oxidative damage. In fact, the heterogeneity of the brain, where certain areas are sensitive to ROS and others are protected by stimulation of redox signalling, could explain the failure of antioxidant strategies to extend lifespan or protect against age-related diseases [7].

3. Mitochondrial ROS during aging.

3.1 Damaged mitochondria that produce more ROS accumulate during aging.

Accumulation of damaged mitochondria is considered an universal hallmark of aging [5]. Mitochondria from brains of old individuals consume less oxygen and generate less ATP [57, 58]. Surprisingly, not all respiratory complexes are affected equally by aging. The activity of complex IV [57, 59] and the activity and levels of CI [14, 49, 60] have been reported by many studies to be reduced, whereas changes in the activity/levels of other respiratory complexes are inconsistent. The reduction in the activity of specific respiratory complexes or the alteration in the ratio between them will have important consequences on ROS levels. Reduction in complex IV activity would increase the redox state of the ETC, stimulating electron leak, ROS production and oxidative stress. On the other hand, reduction in the levels and activity of CI would reduce site-specific ROS signalling produced via CI (see section 2.2).

The accumulation of damaged mitochondria in the brain is usually explained as a consequence of the increased ROS production of brain mitochondria during aging [14, 47, 61, 62]. However, the opposite could be also true, that the increase in mtROS is in fact a consequence of the reduced activity of mitochondria. In fact, data from the "mutator" mouse do not support the usual

interpretation. Due to a knock-in mutation in POLG (*aka* DNA polymerase subunit gamma in charge or replicating and repairing mitochondrial DNA) "mutator" mice accumulate mutations in their mtDNA at an accelerated rate [63]. This results in a strong progeria-like phenotype which includes a significant reduction in mitochondrial respiration [64]. However no increase in mtROS levels [64] or oxidative damage [65] is observed, which does not support the idea that reduction in mitochondrial electron flow increases ROS levels and oxidative damage. In this respect, the "mutator" mice are different to wild type mice where ROS levels [47, 61, 62, 66] in the brain increase in parallel with the accumulation of oxidized lipids[57], proteins[49, 67] and DNA [6, 68]. Therefore, it is also debatable if the "mutator mouse" is a good model of physiological aging.

3.2 How can ROS cause damage and contribute to aging? Do ROS induce cell death or cellular senescence?

ROS can cause damage through two different mechanisms (Figure 2). hiROS such as hydroxyl radicals or peroxynitrites can directly attack proteins, lipids and DNA [69]. loROS such as superoxide or hydrogen peroxide are able to produce highly reactive oxygen species when they react with transition metals through the Fenton's and Haber-Weiss reactions. For example, superoxide can attack iron-sulphur clusters synthetized in the mitochondria and present in many mitochondrial enzymes including aconitase, CI and CII. This causes the release of free iron and hydrogen peroxide leading to the production of hydroxyl radicals, oxidative damage and cell death [70]. Although antioxidants and repair systems are in place to prevent oxidative damage, the fact that oxidized molecules accumulate with age in the brain (see above) demonstrates that these systems are not 100% effective. Accordingly, levels of oxidative damage in the brain correlate with performance in different cognitive and activity tests in rodents [54] suggesting that this type of damage has an impact at a physiological level. Secondly, alterations in site-specific ROS signalling (caused by alterations in complex I and IV) can alter cellular responses affecting mechanisms of quality control or reprogramming cells in ways that make them more vulnerable to cell death [71], senescence [72] or induce their transformation into cancer cells [73]. Finally, both processes are probably interconnected since deregulation of ROS signalling triggers oxidative stress [74] and oxidative stress causes alterations in ROS signalling [75].

Neuronal death is considered the main driver of aging in the brain and the most important initiator of several neurodegenerative diseases [76]. Historically glia have not received the same attention as neurons, recently however it has become evident that they are as important, if not more, as neurons in the aging process [77]. A recent study demonstrated that changes in glia specific genes are more pronounced than changes in neuron specific genes during aging [78], indicating the importance of genomic alterations in glia. Interestingly, changes in gene expression were higher in areas such as the hippocampus and substantia nigra, with higher levels of ROS and oxidative damage as we have discussed previously (section 2.5). Cell death can be caused by mitochondrial dysfunction due to a severe reduction in ATP production or the release of pro-apoptotic factors such as cytochrome c or AIF (Apoptosis Inducing Factor) [79] . Similarly high levels of mtROS can cause cell death via apoptosis or necrosis [80]. In addition to directly promoting cell death in neurons and glia, the age related increase in mtROS promotes neuroinflammation, which can cause neurodegeneration [81]. In this case, the age related increase in mtROS in microglia will cause the secretion of pro-inflammatory cytokines promoting neurodegeneration [81].

Other than inducing cell death mtROS can induce a permanent cell-cycle arrest known as cellular senescence. Senescent cells are characterized by the secretion of pro-inflammatory factors that contribute to the transformation or induction of senescence in neighbouring cells [82]. mtROS are instrumental for the initiation and maintenance of cellular senescence [83, 84]. Maintenance of senescence is achieved via a positive feedback loop where mtROS causes DNA damage (in the nuclear DNA) and activation of CDK1N1 (p21). Subsequently, p21 induces mitochondrial dysfunction and more ROS that are required to maintain DNA damage and senescence [84]. The existence of such a mechanism indicates that mtROS act as redox messengers and not by indiscriminately increasing oxidative damage. Cellular senescence has been considered a mechanism which affects only mitotic cells and therefore would be restricted to glia in the brain. However, a recent pioneering study showed that neurons can also display a senescence-like phenotype that is dependent on p21 and levels of mtROS [72]. Interestingly, the areas of the brain with higher levels of ROS, such as the hippocampus, are also the areas with an increased presence of neurons presenting a senescence-like phenotype [72]. If the existence and importance of senescence cells in the brain is further confirmed, the existence of senolytic drugs that have been shown effective to correct age-related alterations in other organs such as the liver [85] or lung [86] could also be effective in the brain and improve memory or coordination of aged individuals.

3.3 How do ROS increased during ageing?

The "vicious cycle" hypothesis explains the increase in mtROS observed during aging as a consequence of a negative feedback mechanism where mtROS cause the accumulation of mutations in mtDNA (Figure 3A). Mutations in ETC subunits will result in the assembly of defective respiratory complexes that will produce more ROS [87]. Although, this hypothesis has a strong internal logic, it is not fully supported by experimental data. Firstly, mutations found in old individuals (particularly deletions) are more likely caused by errors produced during replication or repair of the mitochondrial DNA than directly by the action of free radicals [88, 89]. Secondly, inducing mutations in mitochondrial DNA does not increase ROS levels in isolated mitochondria [64], although a recent publication measuring mtROS levels *in vivo* found higher levels of ROS in old "mutator" mice when compared to controls [90]. The fact that no differences in ROS were found between young "mutator" mice and controls discards the hypothesis that mutations *per se* are responsible for increased ROS, since young mice already carry more mutations in their mitochondrial DNA than old wild type mice [91]. Finally, interruption of electron flow in mice through mutation of COQ7, an essential gene for the synthesis of CoQ, causes the accumulation of respiratory deficient mitochondria, a progeria-like phenotype and shortens lifespan without increasing ROS levels in isolated mitochondria [92], demonstrating in a different model that interruption of electron flow does not necessarily increase electron leak.

Damaged mitochondria, whole organelles or components, are turned over with the help of quality control mechanisms like autophagy and the proteasome. The age-associated reduction in the efficiency of both mechanisms [5] might explain the accumulation of respiratory-deficient mitochondria during aging (Figure 3B). Supporting this hypothesis, boosting autophagy or increasing the activity of the proteasome extends lifespan in model organisms, including mouse models [93-96]. Moreover, specifically boosting mitophagy i.e. the specialized autophagy process that recycles damaged mitochondria, is enough to extend lifespan in worms and flies [97, 98]. Although these results do not prove that damaged mitochondria *per se* are responsible for aging (see also below), they strongly suggest that maintaining a healthy population of mitochondria in aged individuals is important to guarantee healthy lifespan. Damaged mitochondria that produce higher levels of ROS

can contribute to the collapse of quality control mechanisms in old individuals. Different reports have shown that the efficiency of cellular quality control is regulated by redox changes in key components such as the 20S proteasome [99] or the cysteine protease ATG4 [100]. Very high levels of ROS can cause irreversible oxidation of cysteine residues that are redox regulated impeding the proteins carrying them from executing their normal function. Although published reports are contradictory about whether ROS stimulate or inhibit quality control mechanisms (summarized in [101, 102]), redox regulated control via site-specific ROS signalling would involve specific ROS (e.g. hydrogen peroxide) produced in specific places and amounts. Conversely, damaged mitochondria could generate incorrect signals i.e. the wrong ROS produced at the wrong time/place will prevent the correct function of quality control. If this mechanism is true, the question to answer is why mechanisms of quality control fail in the first place and the answer is probably in the genome.

Since all the information required for making the cell work, and by extension the whole organism, is encoded in the DNA, it is worth looking at how age-related changes in the genome and epigenome affect mitochondria. Recent publications have shown that changes in mitochondrial function are instrumental for cellular differentiation [103]. For example, neuronal differentiation requires a switch from glycolysis to oxidative phosphorylation [104]. Conversely, cellular reprograming of mouse embryonic fibroblasts into pluripotent cells requires repression of oxidative phosphorylation and activation of glycolysis [105]. As we mentioned previously, mitochondrial morphology and composition depends on the amount of ATP and accessory functions mitochondria are required to perform (section 2.4). These characteristics determine how much the mitochondria respire and how many ROS are generated. A very clear example of this is the obvious differences between mitochondria in differentiated and undifferentiated cells. Pluripotent cells have mitochondria with lower rates of respiration, fewer cristae and a rounder morphology [106] when compared to differentiated cells. In parallel with increased oxidative phosphorylation, cell differentiation occurs after a boost in ROS levels [107]. As mentioned previously the information to build both types of mitochondria is encoded in the genome. Aging is characterized by the accumulation of genetic and epigenetic changes that affect both the information encoded in the DNA as well as how this information is interpreted, i.e. how is transcribed and translated [5]. Therefore it is plausible that genetic and epigenetic changes occurring during aging will impact on the nature of the mitochondria present in aged tissues. This could result in the presence of mitochondria that are not "suited" to perform the specific tasks required in a particular cell or location. These "misplaced" mitochondria could generate more ROS in order to activate their own turnover since the mitochondria have mechanisms to sense dysfunction (e.g. RET [19]). Chronically high levels of ROS could potentially trigger oxidative stress and contribute to the saturation of quality control mechanisms, which would spread the damage [33].

Although, the above explanation is only hypothetical, there is preliminary data suggesting that age-related genomic/epigenomic dysfunction could be important in explaining age-related mitochondrial dysfunction warranting further investigation in the future (Figure 3C). Mutations in proteins involved in DNA repair or maintenance cause DNA damage, underlie progeria and have a strong effect on mitochondrial function [108]. Chronic DNA damage, due to defective DNA repair, causes over-activation of repair systems such as PARP or SIRT1 that use NAD+ as a cofactor causing its depletion at the cellular level [109]. Depletion of NAD+ has important metabolic consequences including suppression of mitophagy and the accumulation of damaged mitochondria that produce more ROS [109]. Interestingly, depletion in NAD+ levels has been observed during normal aging [110], whereas supplementing NAD+ or boosting its endogenous synthesis increases mitochondrial function and protects against progeria syndromes as well as physiological aging in worms, flies and

mice [111-113]. Based on the previously described experimental evidence, it is possible that age-related increases in DNA damage [72, 83] activate mechanisms of repair that use NAD+ as cofactor causing its depletion. This could be partially responsible for the increase in damaged mitochondria that produce more ROS.

Another alternative to the "classical vicious cycle" hypothesis is that the increase in mitochondrial ROS is caused by a "ROS induce ROS" mechanism [26] due to increase in ROS produced elsewhere (Figure 3D). Several papers have described the connection between different ROS generators [114]. Particularly significant is the connection between NOXs enzymes and mtROS levels that seems to work in both directions. For example, starvation induces production of mtROS in human 293T cells activating the generation of ROS by NOX1 and cell death if prolonged [115]. Interestingly, mitochondrial ROS are insufficient to cause cell death and requires activation of NOX1 [115]. Conversely, hypoxia induces activation of NOX2 which inhibits CI and increases mtROS in rat pheochromocytoma-12 cells (PC12) [116]. You would expect that there is coordination between specific ROS generators that are part of ROS signalling to make it efficient. For example, if all ROS generators produce ROS simultaneously the background (noise) would be so high that it would prevent the detection of a specific ROS signal. We can speculate that during aging there is an increase in unspecific ROS and a replacement of loROS (such as hydrogen peroxide) with hiROS (such as hydroxyl radical). These changes would "force" site-specific ROS generators in the mitochondria to increase the intensity of the signal establishing a negative cycle that would result in more and more ROS over time. Therefore one of the caveats of using ROS as a signalling system would be the need to increase the intensity of the signal during aging as a result of the accumulation of other types of damage (e.g. epigenetic alterations). Increasing the intensity of the signal would increase the noise and the possibility of triggering oxidative damage.

In summary, the increase in mtROS observed during aging could either be ROS produced as by-products (i.e. the wrong type or amount or produced at the wrong place/time) or a cellular response to the loss of cellular homeostasis associated with aging. In order to find effective anti-aging therapies it is imperative that we understand the exact nature of the ROS (which type, where, when and in which amount) as well as in which cell types and brain regions intervention is required.

4. Conclusions.

4.1 Are increased levels of mtROS a cause or a consequence of aging?

As we have seen, COQ7mutations in mice cause a decrease in mitochondrial respiration and strongly reduce lifespan without increasing mtROS levels [92]. Interestingly, the defect in CoQ synthesis can be rescued by feeding of 4-dihydroxybenzoic acid (2,4-DHB) that bypasses the CoQ synthesis defect and restores mitochondrial respiration. Surprisingly, administration of 2,4-DHB to mutant mice, just before controls fed with the placebo begin to die, was sufficient to restore lifespan [92]. This result is enormously surprising and indicates that the effects of carrying respiratory-deficient mitochondria for more than 1/3 the lifespan in a mouse can be reversed. These results contradict the mitochondrial theory of aging [117] that postulates that loss of mitochondrial function is caused by mutations in mtDNA and independent of ROS levels causes aging. These data indicate that mitochondrial dysfunction is probably more a cause than a consequence of aging. These experiments require replication in other mitochondrial mutants and model organisms but indicate that preventing the accumulation of damaged mitochondria may not be enough to extend lifespan if other

hallmarks of aging (e.g. epigenetic changes or maintenance of quality control mechanisms) are not also targeted. Of course, and since mitochondria from COQ7 mutant mice do not produce more ROS it could be argued that higher levels of mtROS generation are required to cause irreversible damage.

In fact, we have cited many reports demonstrating an increase in mtROS and an accompanying accumulation of oxidative damage in the brain of old individuals (section 3.1). However, it remains to be elucidated whether this is a cause or consequence of aging. Data in lower organisms do not support that increasing ROS accelerates aging. In fact many different reports mainly in worms and flies show that boosting ROS levels can in fact extend lifespan [8]. This phenomenon has been explained as a specific type of hormesis, called mitohormesis [118]. Inducing low or moderate levels of oxidative stress would activate repair and protective mechanisms that long-term can decrease oxidative stress [119]. However, it is not clear if long-term reduction of ROS is required for lifespan extension. For example, overexpression or feeding of antioxidants prevents the positive effect of mitohormesis on lifespan [14, 120, 121]. Moreover, overexpression or feeding of antioxidants, without boosting ROS do not extend lifespan [7]. These results do not support a major role of antioxidant systems in the lifespan extension conferred by mitohormesis, but other repair or protective systems such as autophagy and the proteasome cannot be discarded. ROS can become problematic in old individuals due to the failure in quality control mechanisms that occur during aging [5]. In this context, mtROS could trigger oxidative damage and/or alter redox signalling. From this point of view, the increase in mtROS would initially be a consequence of aging later becoming an important contributor to its progression and may be the cause of the onset of age-related diseases including neurodegenerative diseases such as Parkinson's or Alzheimer's.

4.2 Conclusions: summary.

In summary, available results indicate that high levels of mtROS *per se* are not likely to be the proximal cause of aging, although in combination with other factors (e.g. epigenetic alterations or reduction in quality control systems) can be important for regulating the aging rate and the onset of age-related diseases including neurodegenerative diseases such as Parkinson's. On the other hand, the increase in mtROS could be a signal for the activation of protection mechanisms in middle age. The continuous use of this strategy could cause oxidative stress in later life. Although a consequence and not a cause of aging, this oxidative stress would be still an important contributor to the development of neurodegenerative disorders such as Alzheimer's or Parkinson's disease, decreasing the quality of life and shortening healthy aging.

The present consensus states that low levels of ROS are beneficial, participating in cellular signalling and contributing to homeostasis, while higher levels are deleterious, causing oxidative damage. It is also currently accepted that accumulation of defective mitochondria during ageing is a consequence of increased oxidative stress. Unfortunately, this model does not explain a lot of the published observations, for example the failure of antioxidant therapies to attenuate ageing or that increasing ROS levels can extend lifespan. We propose an alternative model in which specific generators of ROS, such as the mitochondrial site-specific ROS generators CI and III, operate in a coordinated manner to fine tune metabolism and maintain cellular homeostasis in young individuals via redox signalling. During ageing, these signalling pathways, like many others, would be dysregulated resulting in production of unspecific ROS causing oxidative stress. If this model is correct, it is vital we understand how site-specific ROS signalling fails during ageing in order to identify and implement strategies that preserve this signalling and mitochondrial function, extending lifespan.

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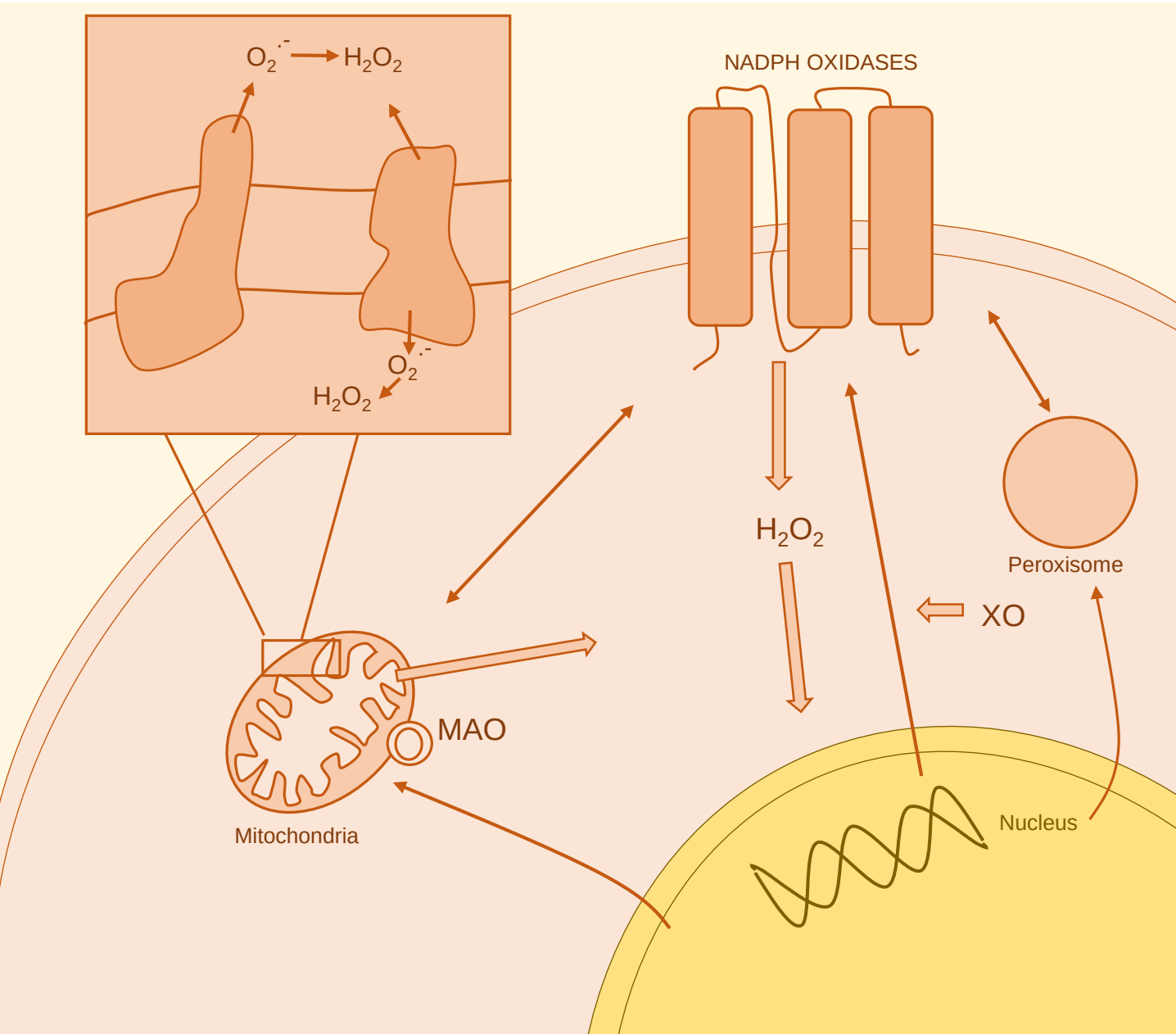
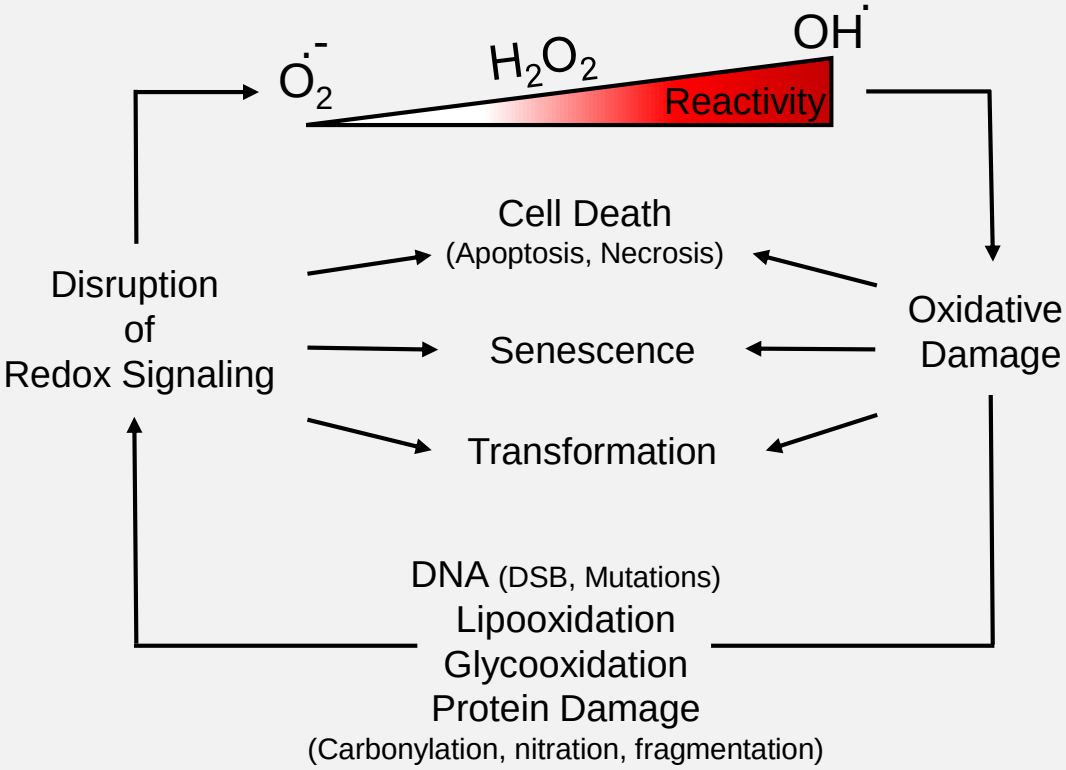
Figure Legends

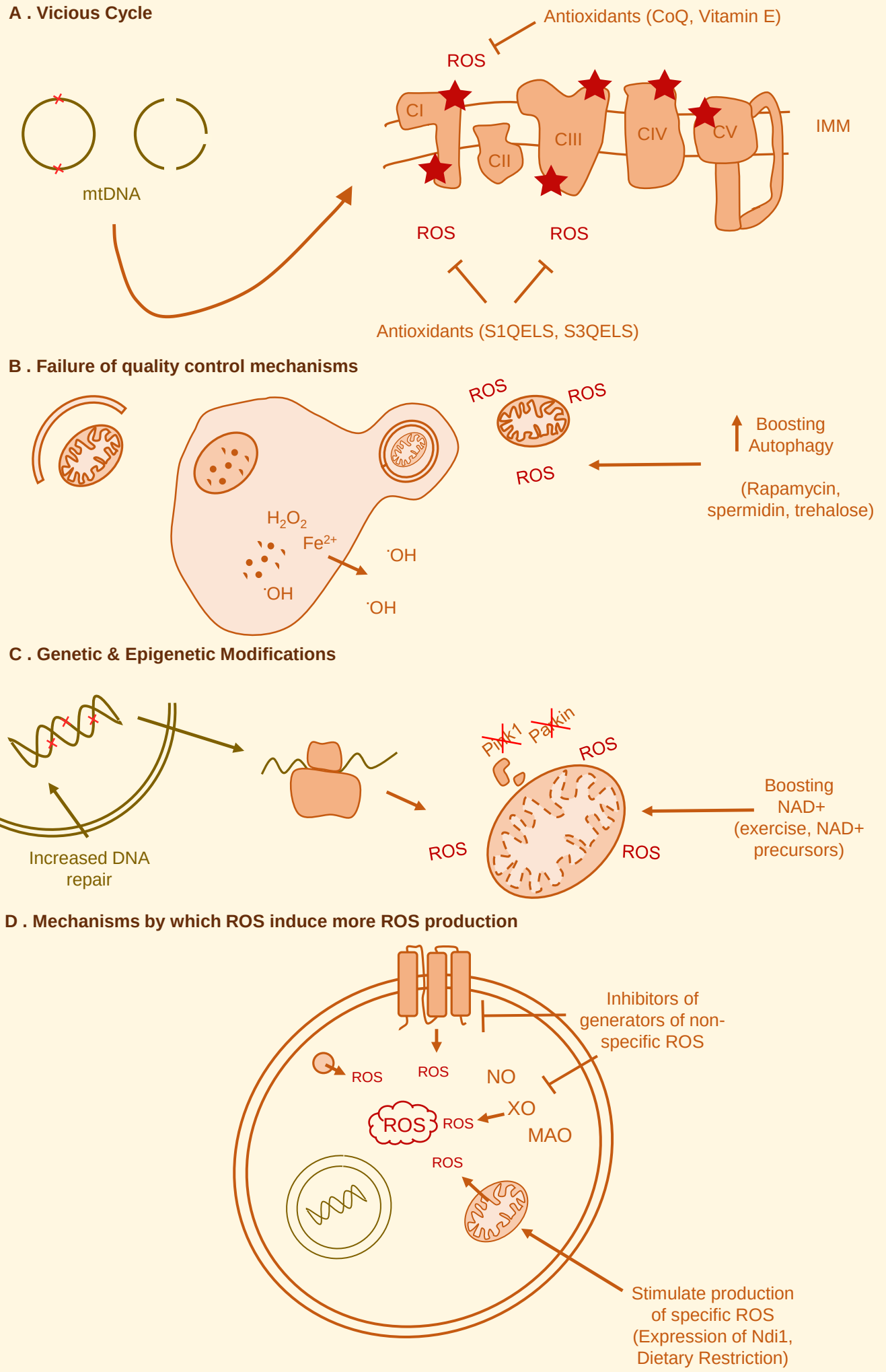
Figure 1. Generators of specific ROS involved in redox signalling. This schematic highlights the sources of ROS involved in redox signalling and their location in the cell. At the cell membrane, we find NADPH oxidases able to produce superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). The peroxisomes and mitochondria are also sites of significant ROS production. Xanthine Oxidase (XO) is located in the cytosol, while Monoamino oxidase (MAO) is located in the mitochondrial outer membrane. Thick arrows highlight the intracellular signalling that specific ROS carry out in order to maintain redox homeostasis which may be lost in ageing. Thin arrows describe the crosstalk between ROS producers and the nucleus in order to regulate redox signalling outputs.

Figure 2. Mechanisms by which ROS can cause damage. As the balance between and levels of more and less reactive ROS are altered in the aging brain, redox signalling is disrupted leading to transformation, senescence and cell death due to a failure to maintain redox homeostasis and increased oxidative damage. This leads to further dysregulation, amplifying the production of ROS from specific and unspecific sources.

Figure 3. Alternative explanations for age associated increases in ROS and potential anti-ageing strategies. The amplification of mtROS with age could be a result of a vicious cycle where ROS produced by the ETC leads to mutations in mtDNA which result in an ETC which produces higher levels of ROS (red stars indicate presence of mutated ETC subunits). Strategies to combat this include the use of antioxidants which can quench ROS before they can induce damage or of new molecules (such as S1QELS and S3QELS) that specifically prevent electron leak at CI or CIII (A). On the other hand, with age the failure or declining efficiency of quality control mechanisms could lead to the lack of or improper clearance of damaged organelles resulting in the accumulation of damaged mitochondria producing increased amounts of ROS as well as the release of reactive metals into the cytosol. This could be counteracted via an enhancement in autophagic clearance of these mitochondria (B). At the DNA level, accumulation of mutations could lead to altered gene expression via changes to the genome and epigenome. The accompanying cellular imbalance could result in inhibition of mitophagy due to the excessive consumption of NAD+ in DNA repair processes. Strategies which prevent DNA damage or boost NAD+ levels would alleviate the accumulation of defective mitochondria (C). Finally, ROS could drive the production of more ROS from sites of unspecific ROS production leading to an increase in the level of site-specific ROS production in order to increase signal intensity. Strategies which repress production of non-signaling ROS while concomitantly increasing the production of signaling ROS has the potential to maintain redox homeostasis and preserve cellular function (D).


$O_2^{\cdot -} \rightarrow H_2O_2$
$O_2^{\cdot -}$
H_2O_2
NADPH OXIDASES
H_2O_2
Peroxisome
XO
MAO
Mitochondria
Nucleus


$O_2^{\cdot -}$
H_2O_2
$OH^{\cdot}$
Reactivity
Cell Death
(Apoptosis, Necrosis)
Disruption
of
Redox Signaling
Senescence
Oxidative
Damage
Transformation
DNA (DSB, Mutations)
Lipooxidation
Glycooxidation
Protein Damage
(Carbonylation, nitration, fragmentation)


A . Vicious Cycle
Antioxidants (CoQ, Vitamin E)
ROS
CI
CII
CIII
CIV
CV
IMM
mtDNA
ROS
ROS
Antioxidants (S1QELS, S3QELS)
B . Failure of quality control mechanisms
ROS
ROS
ROS
Boosting Autophagy
(Rapamycin, spermidin, trehalose)
H2O2
Fe2+
·OH
·OH
·OH
C . Genetic & Epigenetic Modifications
Increased DNA repair
PINK1
Parkin
ROS
ROS
ROS
Boosting NAD+ (exercise, NAD+ precursors)
D . Mechanisms by which ROS induce more ROS production
Inhibitors of generators of non-specific ROS
ROS
ROS
NO
XO
MAO
ROS
ROS
ROS
Stimulate production of specific ROS (Expression of Ndi1, Dietary Restriction)